GENETIC ASPECTS OF CHANGES IN *STAPHYLOCOCCUS AUREUS* PRODUCING STRAINS RESISTANT TO VARIOUS CONCENTRATIONS OF PENICILLIN

M. DEMEREC

Carnegie Institution, Cold Spring Harbor, New York

Fastness or resistance to penicillin and to sulfa drugs is known to occur in strains of bacteria which are sensitive to these chemicals; and in numerous instances on record this new property has been produced *in vitro* under experimental conditions. It is known that the degree of resistance can readily be increased by growing bacteria in a medium containing increasingly higher concentrations of such chemicals, and that the resistant strains so developed retain the property of resistance. My interest in this problem was to obtain quantitative data regarding the origin of resistance and to determine whether or not a genetic interpretation could be given to them. The preliminary summary of these results which has been published (Demerec, '45) indicates that the change to penicillin-resistance in *Staphylococcus aureus* is a genetic change comparable to gene mutation.

Experimental procedure.—The penicillin used in these experiments came from a lot of E. R. Squibb and Sons' preparation of sodium salt of penicillin. This was dissolved in phosphate buffer of pH 6, kept in a refrigerator, and added to the culture medium whenever required by the experiments.

In all experiments a single strain of *Staphylococcus aureus* was used—a culture of which, carrying the number 313, had been obtained from the Northern Regional Research Laboratory in Peoria, Illinois. This same strain has been designated as one of the two international standards for assaying penicillin (Veldee, Herwick and Coghill, '45). In order to reduce the genetic variability of the stock, three cultures were made on agar slants by inoculation from a single colony, and, after 24 hours of incubation, were placed in a refrigerator and used daily as the source of inoculum for all experiments.

Bacteria were grown in a broth medium that did not contain penicillin. They came in contact with the penicillin only when tests for resistance were made. Then certain numbers of bacteria were placed in Petri dishes and mixed with a nutrient agar medium containing the desired concentration of penicillin. These cultures were incubated at 37° C. for 48 hours, and after that period of time bacterial colonies appearing in the medium were counted. The long incubation period was necessary because submerged colonies grow much more slowly in a medium containing penicillin than in one without penicillin.

Results obtained by this method are repeatable; similar numbers of surviving colonies were observed when similar samples of bacteria were taken from the same culture and were plated in a medium containing a certain concentration of penicillin.

Resistance of stock strain.—The strain of *Staphylococcus* used in these experiments was affected by various concentrations of penicillin in the manner shown graphically in fig. 1. The curves given in this figure, representing the results of five experiments, show numbers of survivors when bacteria were grown in penicillin. These six curves are very similar to one another; the heavy line drawn through them represents an average curve.

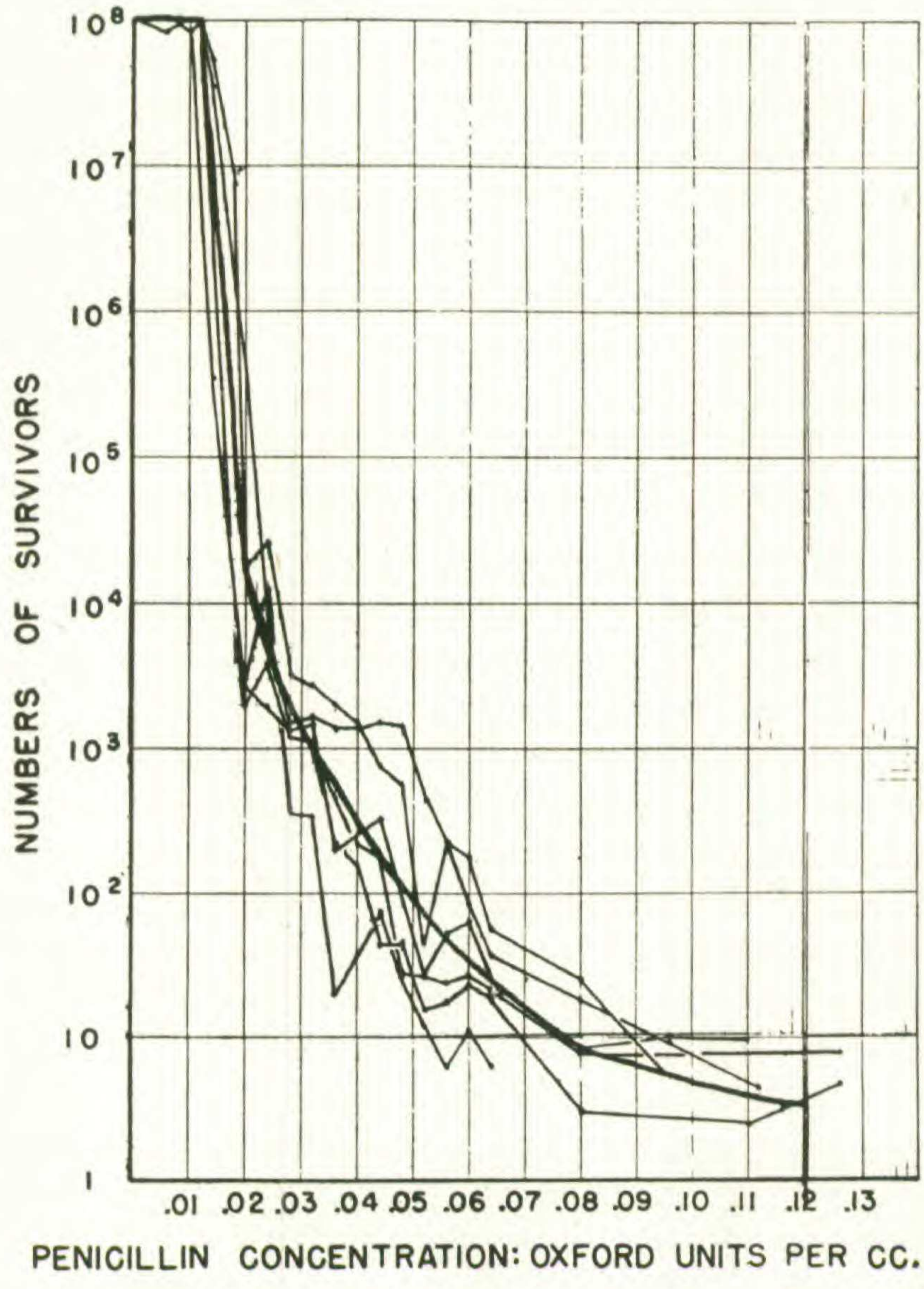

Fig. 1. Numbers of surviving *Staphylococcus aureus* after plating in nutrient agar containing various concentrations of penicillin. The six light curves represent data from six experiments, and the average curve is drawn as a heavy line.

It is evident from fig. 1 that penicillin does not affect these bacteria until a concentration of 0.012 Oxford unit per cc. is reached. That seems to be a threshold concentration for our strain of bacteria. If the concentration is increased by only 0.002 unit over the threshold, the number of surviving bacteria is reduced to 10 per cent. Increase by another 0.002 unit reduces the survivors to 1 per cent;

another similar increase brings the number of survivors down to 1 per 1000; and when a concentration of 0.1 unit per cc. is reached there are on the average only 5 survivors per 100,000,000 bacteria.

The next problem that arose in this study was to find out why some bacteria survive and form colonies in the medium containing penicillin while a great majority of their sister bacteria are eliminated. For this purpose, 32 strains were established—each of them isolated from a single colony that had survived a concentration of 0.064 unit per cc. In order to avoid the possibility of their being members of one clone, each of these colonies was taken from a different experiment. At that concentration (0.064 unit), there are only about 25 survivors per 10^8 bacteria. Tests made with these 32 strains showed that all of them were more resistant to penicillin than was the original stock strain. Survival curves indicated that the threshold for the effectiveness of penicillin had shifted (from the concentration of 0.012 unit per cc. which was the threshold for the original strain) to a region around 0.064 unit per cc. in these selected strains. This result justified the conclusion that survivors in the medium containing 0.064 unit of penicillin per cc. lived because they were resistant to that concentration.

TABLE I
RESISTANCE OF STRAINS ISOLATED FROM COLONIES SURVIVING VARIOUS CONCENTRATIONS OF PENICILLIN

Concentration units/cc.	Number of strains	Resistance higher than parent stock	Resistance similar to parent stock
0.064	32	32	0
0.024	20	18	2
0.022	50	26	24
0.018	54	12	42
0.016	53	10	43

Similar tests were made with 20 strains isolated from cultures containing 0.024 unit of penicillin, with 50 strains isolated from 0.022-unit cultures, 54 strains from 0.018-unit cultures, and 53 strains from 0.016-unit cultures. Results of these tests are summarized in Table I. Of the 20 strains from 0.024-unit cultures, 18 were more resistant than the stock strain, while two had the same degree of resistance as the stock strain. Among the strains isolated at the 0.022 concentration, 52 per cent were resistant; and among those isolated at lower concentrations about 20 per cent were resistant. It is evident that a portion of the survivors on concentrations near the threshold lived not because they were resistant to these concentrations but for some other reason. A possible explanation for the appearance of these survivors is as follows: that occasionally the strength of the penicillin concentration may be reduced in minute sectors of the medium, owing to some environmental factor, and that in the regions near the threshold concentration such reduction may be sufficient to permit the growth of nonresistant bacteria.

Figure 2 gives survival curves for twelve resistant strains isolated at random at various concentrations of penicillin. The heavy line is the survival curve of the stock strain. Arrows pointing toward it indicate the concentrations at which resistant strains were isolated, and each arrow may be matched up (i. e., solid line, broken line, or dotted line) with the curve of the corresponding resistant strain. It is evident that strains isolated from colonies surviving higher concentrations of penicillin tend to be more resistant than strains isolated from colonies surviving lower concentrations.

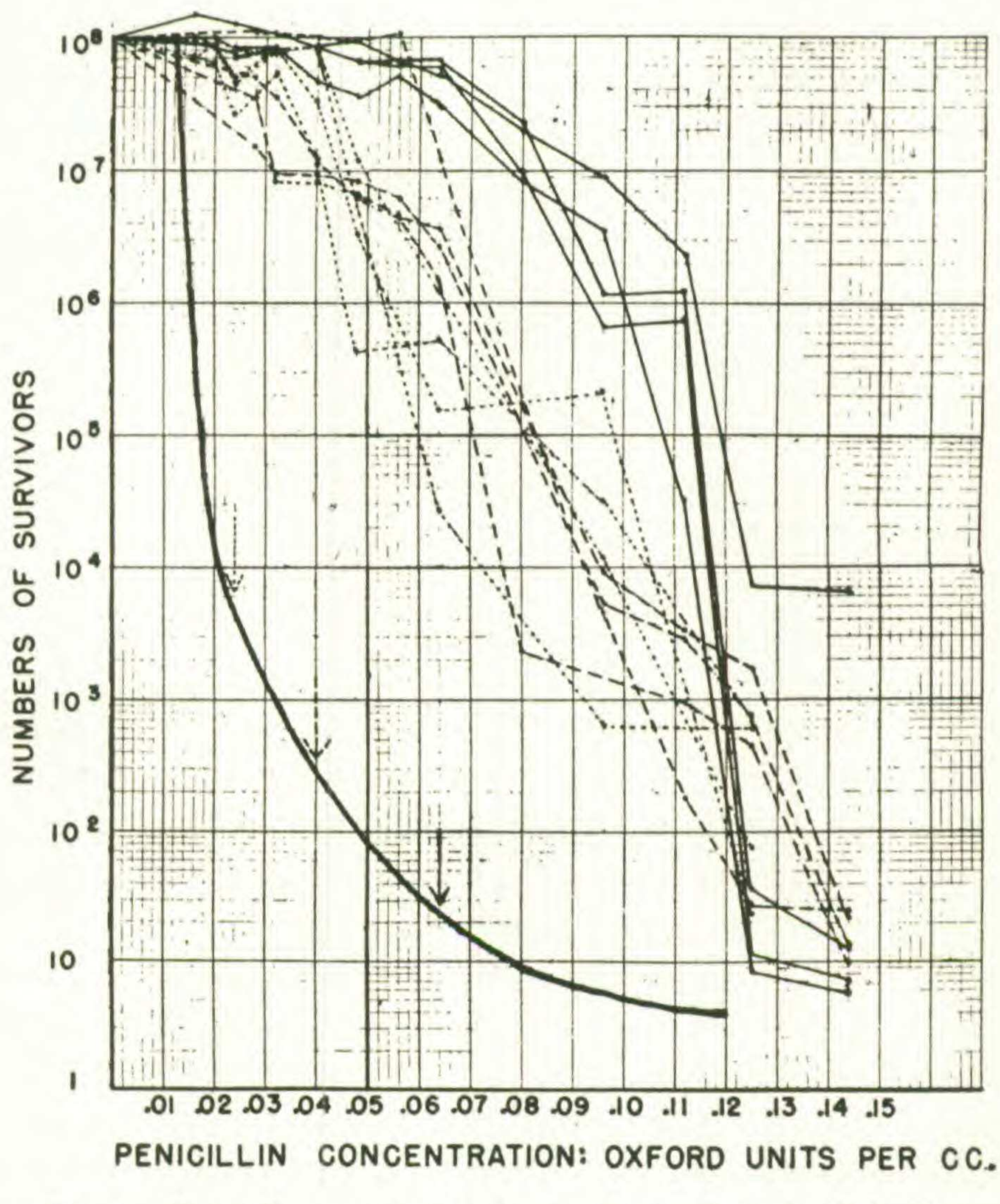

Fig. 2. Heavy line—survival curve for the stock strain; light lines—curves for strains established from colonies surviving the concentrations of penicillin indicated by the arrows.

Inheritance of resistance.—The next problem to be investigated was whether resistance is an inherited characteristic, which persists, or a temporarily acquired feature. Ten strains, isolated from colonies surviving in a concentration of 0.064 unit of penicillin per cc., were passed through 20 transfers in broth; and their survival curves were determined at the beginning of the experiment, at several intervals during the experiment, and at the end of the experiment. The fact that there was no appreciable difference between these curves indicates that the degree of resistance did not change during 20 transfers. Similar results showing the persistance of resistance have been obtained by Schmidt and Sesler ('43) and by Spink, Ferris and Vivino ('44). Thus it is probable that the increase in resistance to penicillin is hereditary, and that it is a stable feature.

Origin of resistance.—The next problem to be investigated dealt with the mechanism of the origin of resistant strains. Here two possibilities were considered: (1) that resistance is an acquired characteristic brought about through some sort of interaction between the bacteria and the penicillin when they are in contact with each other, and (2) that resistance is an inherited characteristic, which originates through mutation and whose origin is independent of penicillin treatment. In the latter case, resistant mutants should occur at random, in a small fraction of a population; and, since a certain concentration of penicillin eliminates all nonresistant individuals, the resistant ones would be selected out from the population by the treatment.

To distinguish between these two possibilities, a modification of the method developed by Luria and Delbrück ('43) in their study of changes in bacteria from bacteriophage-sensitivity to bacteriophage-resistance was used. If the resistance is induced through interaction between the bacteria and penicillin when they are in contact with each other, it would be expected that approximately similar numbers of resistant bacteria would be obtained when samples containing similar numbers of bacteria are plated in nutrient agar containing a certain concentration of penicillin, irrespective of the origin of these samples. The situation would be quite different in the event that the origin of resistance is mutational. In such case, one would expect to obtain similar numbers of resistant colonies only in samples taken from the same culture. If, however, each of the samples comes from a separate culture, and mutations occur at random, then one would expect to obtain a large number of resistant colonies from cultures in which mutation happened to occur early in the growth of the culture and a small number of resistant colonies from cultures in which mutation happened to occur late, assuming that resistant bacteria grow more or less like the normal. If resistance originates by mutation, then, the variation in number of resistant bacteria between samples taken from separate cultures should be much greater than between samples taken from the same culture.

One of the experiments to test these two possibilities was conducted as follows: From the same broth dilution, containing about 300 bacteria per cc., 30 tubes were prepared with 0.3 cc. of material each, and one tube with about 15 cc. of the material. At the same time, 20 samples of 0.3 cc. each from the same dilution were plated in the medium containing 0.064 unit per cc. of penicillin, to determine if any of the samples contained resistant bacteria. None was observed; and therefore it was reasonable to assume that each culture was started with an inoculum consisting of susceptible bacteria only. Cultures were incubated at 37° C. for about 18 hours, and during that time the number of bacteria increased to about 2 x 10^8 per cc.; that is, in the 30 small cultures, from about 100 to about 6.6 x 10^7. The entire contents of each of the 30 tubes were plated in a Petri dish with 0.064 unit of penicillin per cc. of the culture medium; and 20 samples of 0.3 cc. each were taken from the large tube and were similarly plated with the medium containing 0.064 unit of penicillin per cc. In each of these 50 platings about 6.6 x 10^7 bacteria were placed in medium containing an identical concentration of penicillin; therefore, if resistance develops through interaction between bacteria and penicillin, one would expect to find on each plate a similar number of resistant colonies. However, if resistance originates through mutation, then one would expect that the 20 samples taken from the same culture would give similar numbers of resistant colonies, while an appreciable degree of variation in number of resistant colonies would be expected among samples taken from the different cultures. The results (Demerec, '45) show very slight variation in number of resistant colonies among the 20 samples taken from one culture. The extreme variants are 16 and 38; the average number of colonies per culture is 28.9; the variance is 39.8, χ^2 is 22.7, and P is 0.3. On the other hand, the number of resistant colonies per sample taken from independent cultures varies greatly. The extreme variants are 9 and 839, the average is 120, the variance is 42,718, χ^2 is 10,670, and the probability that such a distribution may be due to sampling is extremely small.

The results of this experiment, therefore, favor the assumption that resistance to certain concentrations of penicillin originates through mutation, and that resistant bacteria may be found in any large population. In this case, the proportion of resistant bacteria depends on the mutation rate.

Effect of selection on degree of resistance.—The strain of *Staphylococcus aureus*, NRRL-313, is eliminated if grown in a medium containing more than 0.15 Oxford unit of penicillin per cc. As has been mentioned earlier, an average of 25 out of 10^8 bacteria survived the concentration of 0.064 unit per cc. From these, strains more resistant than the original strain were established. In strains developed from survivors on an 0.125 concentration, there were individuals resistant to 0.25 unit; strains from these latter survivors had individuals resistant to 0.5 unit; strains from these included individuals resistant to 4 units; and from these a strain was isolated that was not affected by a concentration of 250 units of penicillin per cc. of the agar medium.

The result of this type of selection on increase of resistance to penicillin is

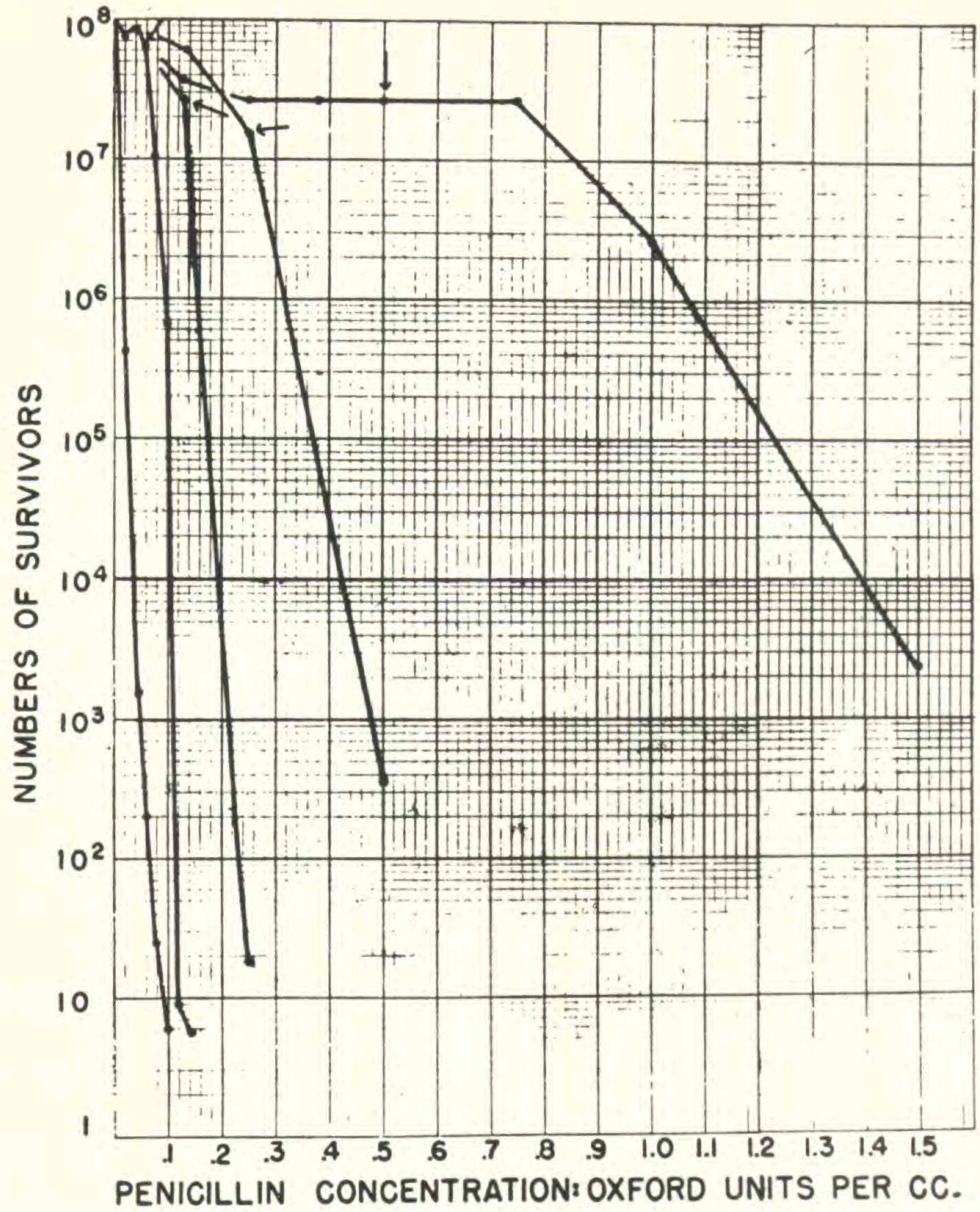

Fig. 3. Numbers of survivors in various concentrations of penicillin, for the stock strain and for four resistant strains developed through repeated selection.

graphically shown in fig. 3. The first curve in this figure represents the number of survivors in various concentrations of penicillin for the stock line of *Staphylococcus;* the second curve represents survivors in a strain isolated from a concentration of 0.064 unit; the third curve shows survivors of a strain isolated from an 0.125 concentration; the fourth is for a strain isolated at an 0.25 concentration; and the fifth for a strain isolated at 0.5 unit. The arrows indicate, on each curve, the concentration at which the strain was isolated. It is evident that the building up of resistance is more rapid with each selection step; the increase appears to be exponential.

Discussion.—The evidence presented here indicates that resistance is a complex characteristic, and that it must involve a number of mutations. If it is assumed that genes are responsible for these mutations, a number of genic changes must be involved. This assumption can readily explain the increase of resistance obtained through selection. A mutation in one of the several genes determining resistance would produce a line having a low degree of resistance. A mutation in another gene, occurring in this line which already had a degree of resistance, would produce a line (double mutant) whose resistance was higher than the sum of the resistances that would be produced by the two mutations if they occurred separately. The increase in resistance caused by another mutation occurring in the double-mutant line would raise it to a degree greater than the sum of resistances produced by the same three mutations occurring separately; and every successive mutation in a multiple-mutant line would produce a similar effect—that is, an increase in resistance proceeding exponentially with the number of mutant genes involved.

Summary.—In experiments with *Staphylococcus aureus,* strains resistant to penicillin were developed which retained that property after 20 transfers in broth.

Experimental evidence indicates that resistance is not induced by the action of penicillin, but originates as a change comparable to mutation. In any large population of bacteria there are some individuals resistant to certain low concentrations of penicillin. If this population is exposed to the action of such concentrations of penicillin, nonresistant individuals are eliminated while the resistant survive.

Degree of resistance can be increased by selection; and this increase is more rapid with each selection step.

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